PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A01H 1/00, 5/00, C12N 15/00
C12N 9/02, C12P 7/64, 1/02
C12P 1/04, 21/06, C07H 15/12
C07H 17/00

A1

(11) International Publication Number: WO 93/06712

(43) International Publication Date: 15 April 1993 (15.04.93)

(21) International Application Number: PCT/US92/08746

(22) International Filing Date: 13 October 1992 (13.10.92)

(30) Priority data:

774,475	10 October 1991 (10.10.91)	US
817,919	8 January 1992 (08.01.92)	US

(60) Parent Application or Grant
(63) Related by Continuation
US 817,919 (CIP)
Filed on 8 January 1992 (08.01.92)

(71) Applicant *(for all designated States except US)*: RHONE-POULENC AGROCHIMIE [FR/FR]; 14-20, rue Pierre-Baizet, B.P. 9163, F-69263 Lyon Cédex 09 (FR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : THOMAS, Terry [US/US]; 3004 Normand, College Station, TX 77845 (US). REDDY, Avutu, S. [IN/US]; 3902 E. 29th Street, #G11, Bryan, TX 77802 (US). NUCCIO, Michael [US/US]; P.O. Box 553, College Station, TX 77841 (US). FREYSSINET, Georges [FR/FR]; 21, rue de Nervleux, F-Saint-Cyr-au-Mont-d'Or (FR).

(74) Agents: SCOTT, Anthony, C. et al.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).

(81) Designated States: AU, BG, BR, CA, CS, HU, JP, KR, PL, RO, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: PRODUCTION OF GAMMA LINOLENIC ACID BY A Δ6-DESATURASE

(57) Abstract

Linoleic acid is converted into γ-linolenic acid by the enzyme Δ6-desaturase. The present invention is directed to an isolated nucleic acid comprising the Δ6-desaturase gene. More particularly, the isolated nucleic acid comprises the promoter, coding region and termination regions of the Δ6-desaturase gene. The present invention provides recombinant constructions comprising the Δ6-desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SK	Slovak Republic
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
UA	Ukraine
US	United States of America
VN	Viet Nam

PRODUCTION OF GAMMA LINOLENIC ACID BY A Δ6-DESATURASE

Linoleic acid (18:2) (LA) is transformed into gamma linolenic acid (18:3) (GLA) by the enzyme Δ6-desaturase. When this enzyme, or the nucleic acid encoding it, is transferred into LA-producing cells, GLA is produced. The present invention provides a nucleic acid comprising the Δ6-desaturase gene. More specifically, the nucleic acid comprises the promoter, coding region and termination regions of the Δ6-desaturase gene. The present invention is further directed to recombinant constructions comprising a Δ6-desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

Unsaturated fatty acids such as linoleic ($C_{18}\Delta^{9,12}$) and α-linolenic ($C_{18}\Delta^{9,12,15}$) acids are essential dietary constituents that cannot be synthesized by vertebrates since vertebrate cells can introduce double bonds at the Δ^9 position of fatty acids but cannot introduce additional double bonds between the Δ^9 double bond and the methyl-terminus of the fatty acid chain. Because they are precursors of other products, linoleic and α-linolenic acids are essential fatty acids, and are usually obtained from plant sources. Linoleic acid can be converted by mammals into γ-linolenic acid (GLA, $C_{18}\Delta^{6,9,12}$) which can in turn be converted to arachidonic acid (20:4), a critically important fatty acid since it is an essential precursor of most prostaglandins.

The dietary provision of linoleic acid, by virtue of its resulting conversion to GLA and arachidonic acid, satisfies the dietary need for GLA and arachidonic acid. However, a relationship has been demonstrated between consumption of saturated fats and health risks such as hypercholesterolemia, atherosclerosis and other chemical disorders which correlate with susceptibility to coronary disease, while the consumption of unsaturated fats has been associated with decreased blood cholesterol concentration and reduced risk of atherosclerosis. The therapeutic benefits of dietary GLA may result from GLA being a precursor to arachidonic acid and thus subsequently contributing to prostaglandin synthesis. Accordingly, consumption of the more unsaturated GLA, rather than linoleic acid, has potential health benefits. However, GLA is not present in virtually any commercially grown crop plant.

Linoleic acid is converted into GLA by the enzyme Δ6-desaturase. Δ6-desaturase, an enzyme of about 359 amino acids, has a membrane-bound domain and an active site for desaturation of fatty acids. When this enzyme is transferred into cells which endogenously produce linoleic acid but not GLA, GLA is produced. The present invention, by providing the gene encoding Δ6-desaturase, allows the production of transgenic organisms which contain functional Δ6-desaturase and which produce GLA. In addition to allowing production of large amounts of GLA, the present invention provides new dietary sources of GLA.

The present invention is directed to an isolated Δ6-desaturase gene. Specifically, the isolated gene

comprises the Δ6-desaturase promoter, coding region, and termination region.

The present invention is further directed to expression vectors comprising the Δ6-desaturase promoter, coding region and termination region.

The present invention is also directed to expression vectors comprising a Δ6-desaturase coding region in functional combination with heterologous regulatory regions, i.e. elements not derived from the Δ6-desaturase gene.

Cells and organisms comprising the vectors of the present invention, and progeny of such organisms, are also provided by the present invention.

The present invention further provides isolated bacterial Δ6-desaturase and is still further directed to an isolated nucleic acid encoding bacterial Δ6-desaturase.

The present invention further provides a method for producing plants with increased gamma linolenic acid (GLA) content which comprises transforming a plant cell with an isolated nucleic acid of the present invention and regenerating a plant with increased GLA content from said plant cell.

A method for producing chilling tolerant plants is also provided by the present invention.

Fig. 1 depicts the hydropathy profiles of the deduced amino acid sequences of Synechocystis Δ6-desaturase (Panel A) and Δ12-desaturase (Panel B). Putative membrane spanning regions are indicated by solid bars. Hydrophobic index was calculated for a window size of 19 amino acid residues [Kyte, et al. (1982) J. Molec. Biol. 157].

Fig. 2 provides gas liquid chromatography profiles of wild type (Panel A) and transgenic (Panel B) Anabaena.

Fig. 3 is a diagram of maps of cosmid cSy75, cSy13 and cSy7 with overlapping regions and subclones. The origins of subclones of cSy75, cSy75-3.5 and cSy7 are indicated by the dashed diagonal lines. Restriction sites that have been inactivated are in parentheses.

Fig. 4 provides gas liquid chromatography profiles of wild type (Panel A) and transgenic (Panel B) tobacco.

The present invention provides an isolated nucleic acid encoding Δ6-desaturase. To identify a nucleic acid encoding Δ6-desaturase, DNA is isolated from an organism which produces GLA. Said organism can be, for example, an animal cell, certain fungi (e.g. Mortierella), certain bacteria (e.g. Synechocystis) or certain plants (borage, Oenothera, currants). The isolation of genomic DNA can be accomplished by a variety of methods well-known to one of ordinary skill in the art, as exemplified by Sambrook et al. (1989) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY. The isolated DNA is fragmented by physical methods or enzymatic digestion and cloned into an appropriate vector, e.g. a bacteriophage or cosmid vector, by any of a variety of well-known methods which can be found in references such as Sambrook et al. (1989). Expression vectors containing the DNA of the present invention are specifically contemplated herein. DNA encoding Δ6-desaturase can be identified by gain of function analysis. The vector containing fragmented DNA is transferred, for example by infection,

transconjugation, transfection, into a host organism that produces linoleic acid but not GLA. As used herein, "transformation" refers generally to the incorporation of foreign DNA into a host cell. Methods for introducing recombinant DNA into a host organism are known to one of ordinary skill in the art and can be found, for example, in Sambrook et al. (1989). Production of GLA by these organisms (i.e., gain of function) is assayed, for example by gas chromatography or other methods known to the ordinarily skilled artisan. Organisms which are induced to produce GLA, i.e. have gained function by the introduction of the vector, are identified as expressing DNA encoding Δ6-desaturase, and said DNA is recovered from the organisms. The recovered DNA can again be fragmented, cloned with expression vectors, and functionally assessed by the above procedures to define with more particularity the DNA encoding Δ6-desaturase.

As an example of the present invention, random DNA is isolated from the cyanobacteria Synechocystis Pasteur Culture Collection (PCC) 6803, American Type Culture Collection (ATCC) 27184, cloned into a cosmid vector, and introduced by transconjugation into the GLA-deficient cyanobacterium Anabaena strain PCC 7120, ATCC 27893. Production of GLA from Anabaena linoleic acid is monitored by gas chromatography and the corresponding DNA fragment is isolated.

The isolated DNA is sequenced by methods well-known to one of ordinary skill in the art as found, for example, in Sambrook et al. (1989).

In accordance with the present invention, a DNA comprising a Δ6-desaturase gene has been isolated. More

particularly, a 3.588 kilobase (kb) DNA comprising a Δ6-desaturase gene has been isolated from the cyanobacteria Synechocystis. The nucleotide sequence of the 3.588 kb DNA was determined and is shown in SEQ ID NO:1. Open reading frames defining potential coding regions are present from nucleotide 317 to 1507 and from nucleotide 2002 to 3081. To define the nucleotides responsible for encoding Δ6-desaturase, the 3.588 kb fragment that confers Δ6-desaturase activity is cleaved into two subfragments, each of which contains only one open reading frame. Fragment ORF1 contains nucleotides 1 through 1704, while fragment ORF2 contains nucleotides 1705 through 3588. Each fragment is subcloned in both forward and reverse orientations into a conjugal expression vector (AM542, Wolk et al. [1984] Proc. Natl. Acad. Sci. USA 81, 1561) that contains a cyanobacterial carboxylase promoter. The resulting constructs (i.e. ORF1(F), ORF1(R), ORF2(F) and ORF2(R)] are conjugated to wild-type Anabaena PCC 7120 by standard methods (see, for example, Wolk et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1561). Conjugated cells of Anabaena are identified as Neo^R green colonies on a brown background of dying non-conjugated cells after two weeks of growth on selective media (standard mineral media BG11N + containing 30μg/ml of neomycin according to Rippka et al., (1979) J. Gen Microbiol. 111, 1). The green colonies are selected and grown in selective liquid media (BG11N + with 15μg/ml neomycin). Lipids are extracted by standard methods (e.g. Dahmer et al., (1989) Journal of American Oil Chemical Society 66, 543) from the resulting transconjugants containing the forward and reverse oriented ORF1 and ORF2 constructs.

For comparison, lipids are also extracted from wild-type cultures of Anabaena and Synechocystis. The fatty acid methyl esters are analyzed by gas liquid chromatography (GLC), for example with a Tracor-560 gas liquid chromatograph equipped with a hydrogen flame ionization detector and a capillary column. The results of GLC analysis are shown in Table 1.

Table 1: Occurrence of C18 fatty acids in wild-type and transgenic cyanobacteria

SOURCE	18:0	18:1	18:2	γ18:3	α18:3	18:4
Anabaena (wild type)	+	+	+	-	+	-
Anabaena + ORF1(F)	+	+	+	-	+	-
Anabaena + ORF1(R)	+	+	+	-	+	-
Anabaena + ORF2(F)	+	+	+	+	+	+
Anabaena + ORF2(R)	+	+	+	-	+	-
Synechocystis (wild type)	+	+	+	+	-	-

As assessed by GLC analysis, GLA deficient Anabaena gain the function of GLA production when the construct containing ORF2 in forward orientation is introduced by transconjugation. Transconjugants containing constructs with ORF2 in reverse orientation to the carboxylase promoter, or ORF1 in either orientation, show no GLA production. This analysis demonstrates that the single open reading frame (ORF2) within the 1884 bp fragment encodes Δ6-desaturase. The 1884 bp fragment is shown as SEQ ID NO:3. This is substantiated by the overall similarity of the hydropathy profiles between Δ6-desaturase and Δ12-

desaturase [Wada et al. (1990) Nature 347] as shown in Fig. 1 as (A) and (B), respectively.

Isolated nucleic acids encoding Δ6-desaturase can be identified from other GLA-producing organisms by the gain of function analysis described above, or by nucleic acid hybridization techniques using the isolated nucleic acid which encodes Anabaena Δ6-desaturase as a hybridization probe. Both genomic and cDNA cloning methods are known to the skilled artisan and are contemplated by the present invention. The hybridization probe can comprise the entire DNA sequence disclosed as SEQ. ID NO:1, or a restriction fragment or other DNA fragment thereof, including an oligonucleotide probe. Methods for cloning homologous genes by cross-hybridization are known to the ordinarily skilled artisan and can be found, for example, in Sambrook (1989) and Beltz et al. (1983) Methods in Enzymology 100, 266.

Transgenic organisms which gain the function of GLA production by introduction of DNA encoding Δ-desaturase also gain the function of octadecatetraeonic acid ($18:4\Delta^{6,9,12,15}$) production. Octadecatetraeonic acid is present normally in fish oils and in some plant species of the Boraginaceae family (Craig et al. [1964] J. Amer. Oil Chem. Soc. 41, 209-211; Gross et al. [1976] Can. J. Plant Sci. 56, 659-664). In the transgenic organisms of the present invention, octadecatetraenoic acid results from further desaturation of α-linolenic acid by Δ6-desaturase or desaturation of GLA by Δ15-desaturase.

The 359 amino acids encoded by ORF2, i.e. the open reading frame encoding Δ6-desaturase, are shown as

SEQ. ID NO:2. The present invention further contemplates other nucleotide sequences which encode the amino acids of SEQ ID NO:2. It is within the ken of the ordinarily skilled artisan to identify such sequences which result, for example, from the degeneracy of the genetic code. Furthermore, one of ordinary skill in the art can determine, by the gain of function analysis described hereinabove, smaller subfragments of the 1884 bp fragment containing ORF2 which encode Δ6-desaturase.

The present invention contemplates any such polypeptide fragment of Δ6-desaturase and the nucleic acids therefor which retain activity for converting LA to GLA.

In another aspect of the present invention, a vector containing the 1884 bp fragment or a smaller fragment containing the promoter, coding sequence and termination region of the Δ6-desaturase gene is transferred into an organism, for example, cyanobacteria, in which the Δ6-desaturase promoter and termination regions are functional. Accordingly, organisms producing recombinant Δ6-desaturase are provided by this invention. Yet another aspect of this invention provides isolated Δ6-desaturase, which can be purified from the recombinant organisms by standard methods of protein purification. (For example, see Ausubel et al. [1987] Current Protocols in Molecular Biology, Green Publishing Associates, New York).

Vectors containing DNA encoding Δ6-desaturase are also provided by the present invention. It will be apparent to one of ordinary skill in the art that appropriate vectors can be constructed to direct the expression of the Δ6-desaturase coding sequence in a

variety of organisms. Replicable expression vectors are particularly preferred. Replicable expression vectors as described herein are DNA or RNA molecules engineered for controlled expression of a desired gene, i.e. the Δ6-desaturase gene. Preferably the vectors are plasmids, bacteriophages, cosmids or viruses. Shuttle vectors, e.g. as described by Wolk et al. (1984) Proc. Natl. Acad. Sci. USA, 1561-1565 and Bustos et al. (1991) J. Bacteriol. 174, 7525-7533, are also contemplated in accordance with the present invention. Sambrook et al. (1989), Goeddel, ed. (1990) Methods in Enzymology 185 Academic Press, and Perbal (1988) A Practical Guide to Molecular Cloning, John Wiley and Sons, Inc., provide detailed reviews of vectors into which a nucleic acid encoding the present Δ6-desaturase can be inserted and expressed. Such vectors also contain nucleic acid sequences which can effect expression of nucleic acids encoding Δ6-desaturase. Sequence elements capable of effecting expression of a gene product include promoters, enhancer elements, upstream activating sequences, transcription termination signals and polyadenylation sites. Both constitutive and tissue specific promoters are contemplated. For transformation of plant cells, the cauliflower mosaic virus (CaMV) 35S promoter and promoters which are regulated during plant seed maturation are of particular interest. All such promoter and transcriptional regulatory elements, singly or in combination, are contemplated for use in the present replicable expression vectors and are known to one of ordinary skill in the art. The CaMV 355 promoter is described, for example, by Restrepo et al. (1990)

Plant Cell 2, 987. Genetically engineered and mutated regulatory sequences are also contemplated.

The ordinarily skilled artisan can determine vectors and regulatory elements suitable for expression in a particular host cell. For example, a vector comprising the promoter from the gene encoding the carboxylase of Anabaena operably linked to the coding region of Δ6-desaturase and further operably linked to a termination signal from Synechocystis is appropriate for expression of Δ6-desaturase in cyanobacteria. "Operably linked" in this context means that the promoter and terminator sequences effectively function to regulate transcription. As a further example, a vector appropriate for expression of Δ6-desaturase in transgenic plants can comprise a seed-specific promoter sequence derived from helianthinin, napin, or glycin operably linked to the Δ6-desaturase coding region and further operably linked to a seed termination signal or the nopaline synthase termination signal.

In particular, the helianthinin regulatory elements disclosed in applicant's copending U.S. Application Serial No. 682,354, filed April 8, 1991 and incorporated herein by reference, are contemplated as promoter elements to direct the expression of the Δ6-desaturase of the present invention.

Modifications of the nucleotide sequences or regulatory elements disclosed herein which maintain the functions contemplated herein are within the scope of this invention. Such modifications include insertions, substitutions and deletions, and specifically substitutions which reflect the degeneracy of the genetic code.

Standard techniques for the construction of such hybrid vectors are well-known to those of ordinary skill in the art and can be found in references such as Sambrook et al. (1989), or any of the myriad of laboratory manuals on recombinant DNA technology that are widely available. A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments. It is further contemplated in accordance with the present invention to include in the hybrid vectors other nucleotide sequence elements which facilitate cloning, expression or processing, for example sequences encoding signal peptides, a sequence encoding KDEL, which is required for retention of proteins in the endoplasmic reticulum or sequences encoding transit peptides which direct Δ6-desaturase to the chloroplast. Such sequences are known to one of ordinary skill in the art. An optimized transit peptide is described, for example, by Van den Broeck et al. (1985) Nature 313, 358. Prokaryotic and eukaryotic signal sequences are disclosed, for example, by Michaelis et al. (1982) Ann. Rev. Microbiol. 36, 425.

A further aspect of the instant invention provides organisms other than cyanobacteria which contain the DNA encoding the Δ6-desaturase of the present invention. The transgenic organisms contemplated in accordance with the present invention include bacteria, cyanobacteria, fungi, and plants and animals. The isolated DNA of the present invention can be introduced into the host by methods known in the art, for example infection, transfection, transformation or transconjugation. Techniques for transferring the DNA

of the present invention into such organisms are widely known and provided in references such as Sambrook et al. (1989).

A variety of plant transformation methods are known. The Δ6-desaturase gene can be introduced into plants by a leaf disk transformation-regeneration procedure as described by Horsch et al. (1985) Science 227, 1229. Other methods of transformation, such as protoplast culture (Horsch et al. (1984) Science 223, 496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et al. (1983) Cell 32, 1033) can also be used and are within the scope of this invention. In a preferred embodiment plants are transformed with Agrobacterium-derived vectors. However, other methods are available to insert the Δ6-desaturase gene of the present invention into plant cells. Such alternative methods include biolistic approaches (Klein et al. (1987) Nature 327, 70), electroporation, chemically-induced DNA uptake, and use of viruses or pollen as vectors.

When necessary for the transformation method, the Δ6-desaturase gene of the present invention can be inserted into a plant transformation vector, e.g. the binary vector described by Bevan (1984) Nucleic Acids Res. 12, 8111. Plant transformation vectors can be derived by modifying the natural gene transfer system of Agrobacterium tumefaciens. The natural system comprises large Ti (tumor-inducing)-plasmids containing a large segment, known as T-DNA, which is transferred to transformed plants. Another segment of the Ti plasmid, the vir region, is responsible for T-DNA transfer. The T-DNA region is bordered by terminal repeats. In the modified binary vectors the tumor-inducing genes have

been deleted and the functions of the vir region are utilized to transfer foreign DNA bordered by the T-DNA border sequences. The T-region also contains a selectable marker for antibiotic resistance, and a multiple cloning site for inserting sequences for transfer. Such engineered strains are known as "disarmed" A. tumefaciens strains, and allow the efficient transformation of sequences bordered by the T-region into the nuclear genomes of plants.

Surface-sterilized leaf disks are inoculated with the "disarmed" foreign DNA-containing A. tumefaciens, cultured for two days, and then transferred to antibiotic-containing medium. Transformed shoots are selected after rooting in medium containing the appropriate antibiotic, transferred to soil and regenerated.

Another aspect of the present invention provides transgenic plants or progeny of these plants containing the isolated DNA of the invention. Both monocotyledenous and dicotyledenous plants are contemplated. Plant cells are transformed with the isolated DNA encoding Δ6-desaturase by any of the plant transformation methods described above. The transformed plant cell, usually in a callus culture or leaf disk, is regenerated into a complete transgenic plant by methods well-known to one of ordinary skill in the art (e.g. Horsch et al. (1985) Science 227, 1129). In a preferred embodiment, the transgenic plant is sunflower, oil seed rape, maize, tobacco, peanut or soybean. Since progeny of transformed plants inherit the DNA encoding Δ6-desaturase, seeds or cuttings from transformed plants are used to maintain the transgenic plant line.

The present invention further provides a method for providing transgenic plants with an increased content of GLA. This method includes introducing DNA encoding Δ6-desaturase into plant cells which lack or have low levels of GLA but contain LA, and regenerating plants with increased GLA content from the transgenic cells. In particular, commercially grown crop plants are contemplated as the transgenic organism, including, but not limited to, sunflower, soybean, oil seed rape, maize, peanut and tobacco.

The present invention further provides a method for providing transgenic organisms which contain GLA. This method comprises introducing DNA encoding Δ6-desaturase into an organism which lacks or has low levels of GLA, but contains LA. In another embodiment, the method comprises introducing one or more expression vectors which comprise DNA encoding Δ12-desaturase and Δ6-desaturase into organisms which are deficient in both GLA and LA. Accordingly, organisms deficient in both LA and GLA are induced to produce LA by the expression of Δ12-desaturase, and GLA is then generated due to the expression of Δ6-desaturase. Expression vectors comprising DNA encoding Δ12-desaturase, or Δ12-desaturase and Δ6-desaturase, can be constructed by methods of recombinant technology known to one of ordinary skill in the art (Sambrook et al., 1989) and the published sequence of Δ12-desaturase (Wada et al [1990] Nature (London) 347, 200-203. In addition, it has been discovered in accordance with the present invention that nucleotides 2002-3081 of SEQ. ID NO:1 encode cyanobacterial Δ12-desaturase. Accordingly, this sequence can be used to construct the subject expression

vectors. In particular, commercially grown crop plants are contemplated as the transgenic organism, including, but not limited to, sunflower, soybean, oil seed rape, maize, peanut and tobacco.

The present invention is further directed to a method of inducing chilling tolerance in plants. Chilling sensitivity may be due to phase transition of lipids in cell membranes. Phase transition temperature depends upon the degree of unsaturation of fatty acids in membrane lipids, and thus increasing the degree of unsaturation, for example by introducing Δ6-desaturase to convert LA to GLA, can induce or improve chilling resistance. Accordingly, the present method comprises introducing DNA encoding Δ6-desaturase into a plant cell, and regenerating a plant with improved chilling resistance from said transformed plant cell. In a preferred embodiment, the plant is a sunflower, soybean, oil seed rape, maize, peanut or tobacco plant.

The following examples further illustrate the present invention.

EXAMPLE 1

Strains and Culture Conditions

Synechocystis (PCC 6803, ATCC 27184), Anabaena (PCC 7120, ATCC 27893) and Synechococcus (PCC 7942, ATCC 33912) were grown photoautotrophically at 30°C in BG11N+ medium (Rippka et al. [1979] J. Gen. Microbiol. 111, 1-61) under illumination of incandescent lamps ($60\mu E.m^{-2}.S^{-1}$). Cosmids and plasmids were selected and propagated in Escherichia coli strain DH5α on LB medium supplemented with antibiotics at standard concentrations as described by Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring, New York.

EXAMPLE 2

Construction of Synechocystis Cosmid Genomic Library

Total genomic DNA from Synechocystis (PCC 6803) was partially digested with Sau3A and fractionated on a sucrose gradient (Ausubel et al. [1987] Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York). Fractions containing 30 to 40 kb DNA fragments were selected and ligated into the dephosphorylated BamHI site of the cosmid vector, pDUCA7 (Buikema et al. [1991] J. Bacteriol. 173, 1879-1885). The ligated DNA was packaged in vitro as described by Ausubel et al. (1987), and packaged phage were propagated in E. coli DH5α containing the AvaI and Eco4711 methylase helper plasmid, pRL528 as described by Buikema et al. (1991). A total of 1152 colonies were isolated randomly and maintained individually in twelve 96-well microtiter plates.

EXAMPLE 3

Gain-of-Function Expression of GLA in Anabaena

Anabaena (PCC 7120), a filamentous cyanobacterium, is deficient in GLA but contains significant amounts of linoleic acid, the precursor for GLA (Figure 2; Table 2). The Synechocystis cosmid library described in Example 2 was conjugated into Anabaena (PCC 7120) to identify transconjugants that produce GLA. Anabaena cells were grown to mid-log phase in BG11N+ liquid medium and resuspended in the same medium to a final concentration of approximately $2x10^8$ cells per ml. A mid-log phase culture of E. coli RP4 (Burkardt et al. [1979] J. Gen. Microbiol. 114, 341-348) grown in LB containing ampicillin was washed and resuspended in fresh LB medium. Anabaena and RP4 were then mixed and spread evenly on BG11N+ plates containing 5% LB. The cosmid genomic library was replica plated onto LB plates containing 50 µg/ml kanamycin and 17.5 µg/ml chloramphenicol and was subsequently patched onto BG11N+ plates containing Anabaena and RP4. After 24 hours of incubation at 30°C, 30 µg/ml of neomycin was underlaid; and incubation at 30°C was continued until transconjugants appeared.

Individual transconjugants were isolated after conjugation and grown in 2 ml BG11N+ liquid medium with 15 µg/ml neomycin. Fatty acid methyl esters were prepared from wild type cultures and cultures containing pools of ten transconjugants as follows. Wild type and transgenic cyanobacterial cultures were harvested by centrifugation and washed twice with distilled water. Fatty acid methyl esters were extracted from these cultures as described by Dahmer et al. (1989) J. Amer.

Oil. Chem. Soc. 66, 543-548 and were analyzed by Gas Liquid Chromatography (GLC) using a Tracor-560 equipped with a hydrogen flame ionization detector and capillary column (30 m x 0.25 mm bonded FSOT Superox II, Alltech Associates Inc., IL). Retention times and co-chromatography of standards (obtained from Sigma Chemical Co.) were used for identification of fatty acids. The average fatty acid composition was determined as the ratio of peak area of each C18 fatty acid normalized to an internal standard.

Representative GLC profiles are shown in Fig. 2. C18 fatty acid methyl esters are shown. Peaks were identified by comparing the elution times with known standards of fatty acid methyl esters and were confirmed by gas chromatography-mass spectrometry. Panel A depicts GLC analysis of fatty acids of wild type Anabaena. The arrow indicates the migration time of GLA. Panel B is a GLC profile of fatty acids of transconjugants of Anabaena with pAM542+1.8F. Two GLA producing pools (of 25 pools representing 250 transconjugants) were identified that produced GLA. Individual transconjugants of each GLA positive pool were analyzed for GLA production; two independent transconjugants, AS13 and AS75, one from each pool, were identified which expressed significant levels of GLA and which contained cosmids, cSy13 and cSy75, respectively (Figure 3). The cosmids overlap in a region approximately 7.5 kb in length. A 3.5 kb NheI fragment of cSy75 was recloned in the vector pDUCA7 and transferred to Anabaena resulting in gain-of-function expression of GLA (Table 2).

Two NheI/Hind III subfragments (1.8 and 1.7 kb) of the 3.5 kb Nhe I fragment of cSy75-3.5 were subcloned into "pBLUESCRIPT" (Stratagene) (Figure 3) for sequencing. Standard molecular biology techniques were performed as described by Maniatis et al. (1982) and Ausubel et al. (1987). Dideoxy sequencing (Sanger et al. [1977] Proc. Natl. Acad. Sci. USA 74, 5463-5467) of pBS1.8 was performed with "SEQUENASE" (United States Biochemical) on both strands by using specific oligonucleotide primers synthesized by the Advanced DNA Technologies Laboratory (Biology Department, Texas A & M University). DNA sequence analysis was done with the GCG (Madison, WI) software as described by Devereux et al. (1984) Nucleic Acids Res. 12, 387-395.

Both NheI/HindIII subfragments were transferred into a conjugal expression vector, AM542, in both forward and reverse orientations with respect to a cyanobacterial carboxylase promoter and were introduced into Anabaena by conjugation. Transconjugants containing the 1.8 kb fragment in the forward orientation (AM542-1.8F) produced significant quantities of GLA and octadecatetraenoic acid (Figure 2; Table 2). Transconjugants containing other constructs, either reverse oriented 1.8 kb fragment or forward and reverse oriented 1.7 kb fragment, did not produce detectable levels of GLA (Table 2).

Figure 2 compares the C18 fatty acid profile of an extract from wild type Anabaena (Figure 2A) with that of transgenic Anabaena containing the 1.8 kb fragment of cSy75-3.5 in the forward orientation (Figure 2B). GLC analysis of fatty acid methyl esters from AM542-1.8F revealed a peak with a retention time identical to that

of authentic GLA standard. Analysis of this peak by gas chromatography-mass spectrometry (GC-MS) confirmed that it had the same mass fragmentation pattern as a GLA reference sample. Transgenic Anabaena with altered levels of polyunsaturated fatty acids were similar to wild type in growth rate and morphology.

Table 2

Composition of C18 Fatty Acids in
Wild Type and Trasgenic Cyanobacteria

Strain	Fatty acid (%)					
	18:0	18:1	18:2	18:3 (α)	18:3 (γ)	18:4
Wild type						
Synechocystis (sp.PCC6803)	13.6	4.5	54.5	-	27.3	-
Anabaena (sp.PCC7120)	2.9	24.8	37.1	35.2	-	-
Synechococcus (Sp.PCC7942)	20.6	79.4	-	-	-	-
Anabaena Transconjugants						
cSy75	3.8	24.4	22.3	9.1	27.9	12.5
cSy75-3.5	4.3	27.6	18.1	3.2	40.4	6.4
pAM542-1.8F	4.2	13.9	12.1	19.1	25.4	25.4
pAM542-1.8R	7.7	23.1	38.4	30.8	-	-
pAM542-1.7F	2.8	27.8	36.1	33.3	-	-
pAM542-1.7R	2.8	25.4	42.3	29.6	-	-
Synechococcus Transformants						
pAM854	27.8	72.2	-	-	-	-
pAM854-Δ^{12}	4.0	43.2	46.0	-	-	-
pAM854-Δ^{6}	18.2	81.8	-	-	-	-
pAM854-Δ^{6} & Δ^{12}	42.7	25.3	19.5	-	16.5	-

18:0, stearic acid; 18.1, oleic acid; 18:2, linoleic acid; 18:3(α), α-linolenic acid; 18:3(γ), γ-linolenic acid; 18:4, octadecatetraenoic acid

EXAMPLE 4

Transformation of Synechococcus with Δ6 and Δ12 Desaturase Genes

A third cosmid, cSy7, which contains a Δ12-desaturase gene, was isolated by screening the Synechocystis genomic library with a oligonucleotide synthesized from the published Synechocystis Δ12-desaturase gene sequence (Wada et al. [1990] Nature (London) 347, 200-203). A 1.7 kb AvaI fragment from this cosmid containing the Δ12-desaturase gene was identified and used as a probe to demonstrate that cSy13 not only contains a Δ6-desaturase gene but also a Δ12-desaturase gene (Figure 3). Genomic Southern blot analysis further showed that both the Δ6-and Δ12-desaturase genes are unique in the Synechocystis genome so that both functional genes involved in C18 fatty acid desaturation are linked closely in the Synechocystis genome.

The unicellular cyanobacterium Synechococcus (PCC 7942) is deficient in both linoleic acid and GLA(3). The Δ12 and Δ6-desaturase genes were cloned individually and together into pAM854 (Bustos et al. [1991] J. Bacteriol. 174, 7525-7533), a shuttle vector that contains sequences necessary for the integration of foreign DNA into the genome of Synechococcus (Golden et al. [1987] Methods in Enzymol. 153, 215-231). Synechococcus was transformed with these gene constructs and colonies were selected. Fatty acid methyl esters were extracted from transgenic Synechococcus and analyzed by GLC.

Table 2 shows that the principal fatty acids of wild type Synechococcus are stearic acid (18:0) and

oleic acid (18:1). Synechococcus transformed with pAM854-Δ12 expressed linoleic acid (18:2) in addition to the principal fatty acids. Transformants with pAM854-Δ6 and Δ12 produced both linoleate and GLA (Table 1). These results indicated that Synechococcus containing both Δ12- and Δ6-desaturase genes has gained the capability of introducing a second double bond at the Δ12 position and a third double bond at the Δ6 position of C18 fatty acids. However, no changes in fatty acid composition was observed in the transformant containing pAM854-Δ6, indicating that in the absence of substrate synthesized by the Δ12 desaturase, the Δ6-desaturase is inactive. This experiment further confirms that the 1.8 kb NheI/HindIII fragment (Figure 3) contains both coding and promoter regions of the Synechocystis Δ6-desaturase gene. Transgenic Synechococcus with altered levels of polyunsaturated fatty acids were similar to wild type in growth rate and morphology.

EXAMPLE 5

Nucleotide Sequence of Δ6-Desaturase

The nucleotide sequence of the 1.8 kb fragment of cSy75-3.5 including the functional Δ6-desaturase gene was determined. An open reading frame encoding a polypeptide of 359 amino acids was identified (Figure 4). A Kyte-Doolittle hydropathy analysis (Kyte et al. [1982] J. Mol. Biol. 157, 105-132) identified two regions of hydrophobic amino acids that could represent transmembrane domains (Figure 1A); furthermore, the hydropathic profile of the Δ6-desaturase is similar to that of the Δ12-desaturase gene (Figure 1B; Wada et al.) and Δ9-desaturases (Thiede et al. [1986] J. Biol. Chem. 261, 13230-13235). However, the sequence similarity between the Synechocystis Δ6- and Δ12-desaturases is less than 40% at the nucleotide level and approximately 18% at the amino acid level.

EXAMPLE 6

Transfer of Cyanobacterial Δ^6-Desaturase into Tobacco

The cyanobacterial Δ^6-desaturase gene was mobilized into a plant expression vector and transferred to tobacco using Agrobacterium mediated gene transfer techniques. To ensure that the transferred desaturase is appropriately expressed in leaves and developing seeds and that the desaturase gene product is targeted to the endoplasmic reticulum or the chloroplast, various expression cassettes with Synechocystis Δ-desaturase open reading frame (ORF) were constructed. Components of these cassettes include: (i) a 35S promoter or seed specific promoter derived from the sunflower helianthinin gene to drive Δ^6-desaturase gene expression in all plant tissues or only in developing seeds respectively, (ii) a putative signal peptide either from carrot extensin gene or sunflower helianthinin gene to target newly synthesized Δ^6-desaturase into the ER, (iii) an ER lumen retention signal sequence (KDEL) at the COOH-terminal of the Δ^6-desaturase ORF, and (iv) an optimized transit peptide to target Δ^6 desaturase into the chloroplast. The 35S promoter is a derivative of pRTL2 described by Restrepo et al. (1990). The optimized transit peptide sequence is described by Van de Broeck et al. (1985). The carrot extensin signal peptide is described by Chen et al (1985) EMBO J. 9, 2145.

Transgenic tobacco plants were produced containing a chimeric cyanobacterial desaturase gene, comprised of the Synechocystis Δ^6 desaturase gene fused to an endoplasmic reticulum retention sequence (KDEL) and extensin signal peptide driven by the CaMV 35S promoter. PCR amplifications of transgenic tobacco genomic DNA indicate that the Δ^6 desaturase gene was incorporated into the tobacco genome. Fatty acid methyl esters of leaves of these transgenic tobacco plants were

extracted and analyzed by Gas Liquid Chromatography (GLC). These transgenic tobacco accumulated significant amounts of GLA (Figure 4). Figure 4 shows fatty acid methyl esters as determined by GLC. Peaks were identified by comparing the elution times with known standards of fatty acid methyl ester. Accordingly, cyanobacterial genes involved in fatty acid metabolism can be used to generate transgenic plants with altered fatty acid compositions.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Thomas, Terry L.
Reddy, Avutu S.
Nuccio, Michael
Freyssinet, Georges L.

(ii) TITLE OF INVENTION: PRODUCTION OF GAMMA LINOLENIC ACID BY A DELTA 6-DESATURASE

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Scully, Scott, Murphy & Presser
(B) STREET: 400 Garden City Plaza
(C) CITY: Garden City
(D) STATE: New York
(E) COUNTRY: United States
(F) ZIP: 11530

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: To be assigned
(B) FILING DATE: 08-JAN-1992
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: McNulty, William E.
(B) REGISTRATION NUMBER: 22,606
(C) REFERENCE/DOCKET NUMBER: 8383Z

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (516) 742-4343
(B) TELEFAX: (516) 742-4366
(C) TELEX: 230 901 SANS UR

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3588 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2002..3081

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTAGCCACC AGTGACGATG CCTTGAATTT GGCCATTCTG ACCCAGGCCC GTATTCTGAA 60

TCCCCGCATT CGCATTGTTA ATCGTTTGTT CAACCATGCC CTGGGTAAAC GTTTAGACAC 120

CACCTTGCCA GACCACGTTA GTTTGAGTGT TTCCGCCCTG GCGGCCCCGA TTTTTTCCTT 180

TGCGGCTTTG GGCAATCAGG CGATCGGGCA ATTGCGTTTG TTTGACCAGA CTTGGCCCAT 240

TCAGGAAATT GTCATTCACC AAGACCATCC CTGGCTCAAT TTACCCCTGG CGGATTTATG 300

GGATGATCCG AGCCGAATGT TGATCTATTA CCTACCGGCC CACAGTGAAA CGGATTTAGT 360

AGGCGCAGTG GTGAATAATT TAACGTTGCA ATCTGGGGAC CATTTAATAG TGGGACAAAA 420

ACCCCAACCC AAGACCAAAC GGCGATCGCC TTGGCGCAAA TTTTCCAAAC TGATTACCAA 480

CCTGCGGGAG TATCAGCGGT ATGTCCAACA GGTGATATGG GTGGTGTTGT TTTTATTGTT 540

GATGATTTTT CTGGCCACCT TCATCTACGT TTCCATTGAT CAACATATTG CCCCAGTGGA 600

CGCGTTGTAT TTTTCCGTGG GCATGATTAC CGGGGCCGGT GGCAAGGAAG AGGTGGCCGA 660

AAAGTCCCCC GATATCATCA AAGTATTCAC AGTGGTGATG ATGATCGCCG GGGCGGGGGT 720

GATTGGTATT TGTTATGCCC TACTGAATGA TTTCATCCTT GGCAGTCGCT TTAGTCAGTT 780

TTTGGATGCG GCCAAGTTAC CCGATCGCCA TCACATCATC ATTTGTGGGC TGGGGGGAGT 840

GAGCATGGCC ATTATTGAAG AGTTAATTCA CCAGGGCCAT GAAATTGTGG TAATCGAAAA 900

GGATACAGAT AATCGTTTCT TGCATACGGC CCGCTCCCTG GGGGTGCCCG TAATTGTGGA 960

```
1   GGATGCCCGC CTAGAAAGAA CGTTGGCCTG CGCCAATATC AACCGAGCCG AAGCCATTGT   1020
    GGTGGCCACC AGCGACGACA CCGTTAACTT GGAAATTGGC CTAACTGCCA AGGCGATCGC   1080
    CCCTAGCCTG CCAGTGGTGT TGCGTTGCCA GGATGCCCAG TTTAGCCTGT CCCTGCAGGA   1140
    AGTATTTGAA TTTGAAACGG TGCTTTGTCC GGCGGAATTG GCCACCTATT CCTTTGCGGC   1200
5   GGCGGCCCTG GGGGGCAAAA TTTTGGGCAA CGGCATGACC GATGATTTGC TGTGGGTAGC   1260
    CCTAGCCACC TTAATCACTC CTAACCATCC CTTTGCCGAC CAATTGGTTA AAATTGCAGC   1320
    CCAAAAGTCT GATTTCGTTC CCCTCTATCT AGAACGGGGT GGCAAAACCA TCCATAGCTG   1380
    GGAATTATTG GGTACCCATC TCGACTCTGG AGACGTGTTG TATTTAACCA TGCCCGCCAC   1440
10  TGCCCTAGAG CAACTTTGGC GATCGCCCCG TGCCACTGCT GATCCTCTGG ACTCTTTTTT   1500
    GGTTTAGCAT GGGGGGATGG AACTCTTGAC TCGGCCCAAT GGTGATCAAG AAAGAACGCT   1560
    TTGTCTATGT TTAGTATTTT TAAGTTAACC AACAGCAGAG GATAACTTCC AAAAGAAATT   1620
    AAGCTCAAAA AGTAGCAAAA TAAGTTTAAT TCATAACTGA GTTTTACTGC TAAACAGCGG   1680
    TGCAAAAAAG TCAGATAAAA TAAAAGCTTC ACTTCGGTTT TATATTGTGA CCATGGTTCC   1740
15  CAGGCATCTG CTCTAGGGAG TTTTTCCGCT GCCTTTAGAG AGTATTTTCT CCAAGTCGGC   1800
    TAACTCCCCC ATTTTTAGGC AAAATCATAT ACAGACTATC CCAATATTGC CAGAGCTTTG   1860
    ATGACTCACT GTAGAAGGCA GACTAAAATT CTAGCAATGG ACTCCCAGTT GGAATAAATT   1920
    TTTAGTCTCC CCCGGCGCTG GAGTTTTTTT GTAGTTAATG GCGGTATAAT GTGAAAGTTT   1980
    TTTATCTATT TAAATTTATA A ATG CTA ACA GCG GAA AGA ATT AAA TTT ACC      2031
20                           Met Leu Thr Ala Glu Arg Ile Lys Phe Thr
                               1               5                  10
    CAG AAA CGG GGG TTT CGT CGG GTA CTA AAC CAA CGG GTG GAT GCC TAC     2079
    Gln Lys Arg Gly Phe Arg Arg Val Leu Asn Gln Arg Val Asp Ala Tyr
                      15                  20                  25
    TTT GCC GAG CAT GGC CTG ACC CAA AGG GAT AAT CCC TCC ATG TAT CTG     2127
    Phe Ala Glu His Gly Leu Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu
                30                  35                  40
25
```

30

35

```
AAA ACC CTG ATT ATT GTG CTC TGG TTG TTT TCC GCT TGG GCC TTT GTG      2175
Lys Thr Leu Ile Ile Val Leu Trp Leu Phe Ser Ala Trp Ala Phe Val
          45                  50                  55

CTT TTT GCT CCA GTT ATT TTT CCG GTG CGC CTA CTG GGT TGT ATG GTT      2223
Leu Phe Ala Pro Val Ile Phe Pro Val Arg Leu Leu Gly Cys Met Val
      60                  65                  70

TTG GCG ATC GCC TTG GCG GCC TTT TCC TTC AAT GTC GGC CAC GAT GCC      2271
Leu Ala Ile Ala Leu Ala Ala Phe Ser Phe Asn Val Gly His Asp Ala
 75                  80                  85                  90

AAC CAC AAT GCC TAT TCC TCC AAT CCC CAC ATC AAC CGG GTT CTG GGC      2319
Asn His Asn Ala Tyr Ser Ser Asn Pro His Ile Asn Arg Val Leu Gly
                95                 100                 105

ATG ACC TAC GAT TTT GTC GGG TTA TCT AGT TTT CTT TGG CGC TAT CGC      2367
Met Thr Tyr Asp Phe Val Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg
            110                 115                 120

CAC AAC TAT TTG CAC CAC ACC TAC ACC AAT ATT CTT GGC CAT GAC GTG      2415
His Asn Tyr Leu His His Thr Tyr Thr Asn Ile Leu Gly His Asp Val
        125                 130                 135

GAA ATC CAT GGA GAT GGC GCA GTA CGT ATG AGT CCT GAA CAA GAA CAT      2463
Glu Ile His Gly Asp Gly Ala Val Arg Met Ser Pro Glu Gln Glu His
    140                 145                 150

GTT GGT ATT TAT CGT TTC CAG CAA TTT TAT ATT TGG GGT TTA TAT CTT      2511
Val Gly Ile Tyr Arg Phe Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu
155                 160                 165                 170

TTC ATT CCC TTT TAT TGG TTT CTC TAC GAT GTC TAC CTA GTG CTT AAT      2559
Phe Ile Pro Phe Tyr Trp Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn
                175                 180                 185

AAA GGC AAA TAT CAC GAC CAT AAA ATT CCT CCT TTC CAG CCC CTA GAA      2607
Lys Gly Lys Tyr His Asp His Lys Ile Pro Pro Phe Gln Pro Leu Glu
            190                 195                 200

TTA GCT AGT TTG CTA GGG ATT AAG CTA TTA TGG CTC GGC TAC GTT TTC      2655
Leu Ala Ser Leu Leu Gly Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe
        205                 210                 215

GGC TTA CCT CTG GCT CTG GGC TTT TCC ATT CCT GAA GTA TTA ATT GGT      2703
Gly Leu Pro Leu Ala Leu Gly Phe Ser Ile Pro Glu Val Leu Ile Gly
    220                 225                 230
```

```
GCT TCG GTA ACC TAT ATG ACC TAT GGC ATC GTG GTT TGC ACC ATC TTT      2751
Ala Ser Val Thr Tyr Met Thr Tyr Gly Ile Val Val Cys Thr Ile Phe
235                 240                 245                 250

ATG CTG GCC CAT GTG TTG GAA TCA ACT GAA TTT CTC ACC CCC GAT GGT      2799
Met Leu Ala His Val Leu Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly
                255                 260                 265

GAA TCC GGT GCC ATT GAT GAC GAG TGG GCT ATT TGC CAA ATT CGT ACC      2847
Glu Ser Gly Ala Ile Asp Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr
            270                 275                 280

ACG GCC AAT TTT GCC ACC AAT AAT CCC TTT TGG AAC TGG TTT TGT GGC      2895
Thr Ala Asn Phe Ala Thr Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly
        285                 290                 295

GGT TTA AAT CAC CAA GTT ACC CAC CAT CTT TTC CCC AAT ATT TGT CAT      2943
Gly Leu Asn His Gln Val Thr His His Leu Phe Pro Asn Ile Cys His
    300                 305                 310

ATT CAC TAT CCC CAA TTG GAA AAT ATT ATT AAG GAT GTT TGC CAA GAG      2991
Ile His Tyr Pro Gln Leu Glu Asn Ile Ile Lys Asp Val Cys Gln Glu
315                 320                 325                 330

TTT GGT GTG GAA TAT AAA GTT TAT CCC ACC TTC AAA GCG GCG ATC GCC      3039
Phe Gly Val Glu Tyr Lys Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala
                335                 340                 345

TCT AAC TAT CGC TGG CTA GAG GCC ATG GGC AAA GCA TCG TGACATTGCC       3088
Ser Asn Tyr Arg Trp Leu Glu Ala Met Gly Lys Ala Ser
            350                 355                 360

TTGGGATTGA AGCAAAATGG CAAAATCCCT CGTAAATCTA TGATCGAAGC CTTTCTGTTG    3148

CCCGCCGACC AAATCCCCGA TGCTGACCAA AGGTTGATGT TGGCATTGCT CCAAACCCAC    3208

TTTGAGGGGG TTCATTGGCC GCAGTTTCAA GCTGACCTAG GAGGCAAAGA TTGGGTGATT    3268

TTGCTCAAAT CCGCTGGGAT ATTGAAAGGC TTCACCACCT TTGGTTTCTA CCCTGCTCAA    3328

TGGGAAGGAC AAACCGTCAG AATTGTTTAT TCTGGTGACA CCATCACCGA CCCATCCATG    3388

TGGTCTAACC CAGCCCTGGC CAAGGCTTGG ACCAAGGCCA TGCAAATTCT CCACGAGGCT    3448

AGGCCAGAAA AATTATATTG GCTCCTGATT TCTTCCGGCT ATCGCACCTA CCGATTTTTG    3508

AGCATTTTTG CCAAGGAATT CTATCCCCAC TATCTCCATC CCACTCCCCC GCCTGTACAA    3568
```

AATTTTATCC ATCAGCTAGC 3588

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 359 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Leu Thr Ala Glu Arg Ile Lys Phe Thr Gln Lys Arg Gly Phe Arg
 1               5                  10                  15

Arg Val Leu Asn Gln Arg Val Asp Ala Tyr Phe Ala Glu His Gly Leu
            20                  25                  30

Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu Lys Thr Leu Ile Ile Val
        35                  40                  45

Leu Trp Leu Phe Ser Ala Trp Ala Phe Val Leu Phe Ala Pro Val Ile
    50                  55                  60

Phe Pro Val Arg Leu Leu Gly Cys Met Val Leu Ala Ile Ala Leu Ala
65                  70                  75                  80

Ala Phe Ser Phe Asn Val Gly His Asp Ala Asn His Asn Ala Tyr Ser
                85                  90                  95

Ser Asn Pro His Ile Asn Arg Val Leu Gly Met Thr Tyr Asp Phe Val
            100                 105                 110

Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg His Asn Tyr Leu His His
        115                 120                 125

Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly
    130                 135                 140

Ala Val Arg Met Ser Pro Glu Gln Glu His Val Gly Ile Tyr Arg Phe
145                 150                 155                 160

Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu Phe Ile Pro Phe Tyr Trp
                165                 170                 175
```

Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn Lys Gly Lys Tyr His Asp
180 185 190

His Lys Ile Pro Pro Phe Gln Pro Leu Glu Leu Ala Ser Leu Leu Gly
195 200 205

Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe Gly Leu Pro Leu Ala Leu
210 215 220

Gly Phe Ser Ile Pro Glu Val Leu Ile Gly Ala Ser Val Thr Tyr Met
225 230 235 240

Thr Tyr Gly Ile Val Val Cys Thr Ile Phe Met Leu Ala His Val Leu
245 250 255

Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly Glu Ser Gly Ala Ile Asp
260 265 270

Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr Thr Ala Asn Phe Ala Thr
275 280 285

Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly Gly Leu Asn His Gln Val
290 295 300

Thr His His Leu Phe Pro Asn Ile Cys His Ile His Tyr Pro Gln Leu
305 310 315 320

Glu Asn Ile Ile Lys Asp Val Cys Gln Glu Phe Gly Val Glu Tyr Lys
325 330 335

Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala Ser Asn Tyr Arg Trp Leu
340 345 350

Glu Ala Met Gly Lys Ala Ser
355

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1884 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
1    AGCTTCACTT CGGTTTTATA TTGTGACCAT GGTTCCCAGG CATCTGCTCT AGGGAGTTTT   60
     TCCGCTGCCT TTAGAGAGTA TTTTCTCCAA GTCGGCTAAC TCCCCCATTT TTAGGCAAAA  120
     TCATATACAG ACTATCCCAA TATTGCCAGA GCTTTGATGA CTCACTGTAG AAGGCAGACT  180
     AAAATTCTAG CAATGGACTC CCAGTTGGAA TAAATTTTTA GTCTCCCCCG GCGCTGGAGT  240
5    TTTTTTGTAG TTAATGGCGG TATAATGTGA AAGTTTTTTA TCTATTTAAA TTTATAAATG  300
     CTAACAGCGG AAAGAATTAA ATTTACCCAG AAACGGGGGT TTCGTCGGGT ACTAAACCAA  360
     CGGGTGGATG CCTACTTTGC CGAGCATGGC CTGACCCAAA GGGATAATCC CTCCATGTAT  420
     CTGAAAACCC TGATTATTGT GCTCTGGTTG TTTTCCGCTT GGGCCTTTGT GCTTTTTGCT  480
10   CCAGTTATTT TTCCGGTGCG CCTACTGGGT TGTATGGTTT TGGCGATCGC CTTGGCGGCC  540
     TTTTCCTTCA ATGTCGGCCA CGATGCCAAC CACAATGCCT ATTCCTCCAA TCCCCACATC  600
     AACCGGGTTC TGGGCATGAC CTACGATTTT GTCGGGTTAT CTAGTTTTCT TTGGCGCTAT  660
     CGCCACAACT ATTTGCACCA CACCTACACC AATATTCTTG GCCATGACGT GGAAATCCAT  720
     GGAGATGGCG CAGTACGTAT GAGTCCTGAA CAAGAACATG TTGGTATTTA TCGTTTCCAG  780
15   CAATTTTATA TTTGGGGTTT ATATCTTTTC ATTCCCTTTT ATTGGTTTCT CTACGATGTC  840
     TACCTAGTGC TTAATAAAGG CAAATATCAC GACCATAAAA TTCCTCCTTT CCAGCCCCTA  900
     GAATTAGCTA GTTTGCTAGG GATTAAGCTA TTATGGCTCG GCTACGTTTT CGGCTTACCT  960
     CTGGCTCTGG GCTTTTCCAT TCCTGAAGTA TTAATTGGTG CTTCGGTAAC CTATATGACC 1020
     TATGGCATCG TGGTTTGCAC CATCTTTATG CTGGCCCATG TGTTGGAATC AACTGAATTT 1080
20   CTCACCCCCG ATGGTGAATC CGGTGCCATT GATGACGAGT GGGCTATTTG CCAAATTCGT 1140
     ACCACGGCCA ATTTTGCCAC CAATAATCCC TTTTGGAACT GGTTTTGTGG CGGTTTAAAT 1200
     CACCAAGTTA CCCACCATCT TTTCCCCAAT ATTTGTCATA TTCACTATCC CCAATTGGAA 1260
     AATATTATTA AGGATGTTTG CCAAGAGTTT GGTGTGGAAT ATAAAGTTTA TCCCACCTTC 1320
25   AAAGCGGCGA TCGCCTCTAA CTATCGCTGG CTAGAGGCCA TGGGCAAAGC ATCGTGACAT 1380
     TGCCTTGGGA TTGAAGCAAA ATGGCAAAAT CCCTCGTAAA TCTATGATCG AAGCCTTTCT 1440
```

30

35

GTTGCCCGCC GACCAAATCC CCGATGCTGA CCAAAGGTTG ATGTTGGCAT TGCTCCAAAC 1500

CCACTTTGAG GGGGTTCATT GGCCGCAGTT TCAAGCTGAC CTAGGAGGCA AAGATTGGGT 1560

GATTTTGCTC AAATCCGCTG GGATATTGAA AGGCTTCACC ACCTTTGGTT TCTACCCTGC 1620

TCAATGGGAA GGACAAACCG TCAGAATTGT TTATTCTGGT GACACCATCA CCGACCCATC 1680

CATGTGGTCT AACCCAGCCC TGGCCAAGGC TTGGACCAAG GCCATGCAAA TTCTCCACGA 1740

GGCTAGGCCA GAAAAATTAT ATTGGCTCCT GATTTCTTCC GGCTATCGCA CCTACCGATT 1800

TTTGAGCATT TTTGCCAAGG AATTCTATCC CCACTATCTC CATCCCACTC CCCCGCCTGT 1860

ACAAAATTTT ATCCATCAGC TAGC 1884

WHAT IS CLAIMED:

1. An isolated nucleic acid encoding bacterial Δ6-desaturase.

2. The nucleic acid of Claim 1 comprising the nucleotides of SEQ. ID NO:3.

3. An isolated nucleic acid that codes for the amino acid sequence encoded by the nucleic acid of Claim 1.

4. The isolated nucleic acid of any one of Claims 1-3 wherein said nucleic acid is contained in a vector.

5. The isolated nucleic acid of Claim 4 operably linked to a promoter and/or a termination signal capable of effecting expression of the gene product of said isolated nucleic acid.

6. The isolated nucleic acid of Claim 5 wherein said promoter is a Δ6-desaturase promoter, an Anabaena carboxylase promoter, a helianthinin promoter, a glycin promoter, a napin promoter, or a helianthinin tissue-specific promoter.

7. The isolated nucleic acid of Claim 5 wherein said termination signal is a Synechocystis termination signal, a nopaline synthase termination signal, or a seed termination signal.

8. The isolated nucleic acid of any one of Claims 1-7 wherein said isolated nucleic acid is contained within a transgenic organism.

9. The isolated nucleic acid of Claim 8 wherein said transgenic organism is a bacterium, a fungus, a plant cell or an animal.

10. A plant or progeny of said plant which has been regenerated from the transgenic plant cell of Claim 9.

11. The plant of Claim 10 wherein said plant is a sunflower, soybean, maize, tobacco, peanut or oil seed rape plant.

12. A method of producing a plant with increased gamma linolenic acid (GLA) content which comprises:

(a) transforming a plant cell with the isolated nucleic acid of any one of Claims 1-7; and

(b) regenerating a plant with increased GLA content from said plant cell.

13. The method of Claim 12 wherein said plant is a sunflower, soybean, maize, tobacco, peanut or oil seed rape plant.

14. A method of inducing production of gamma linolenic acid (GLA) in an organism deficient or lacking in GLA with comprises transforming said organism with the isolated nucleic acid of any one of Claims 1-7.

15. A method of inducing production of gamma linolenic acid (GLA) in an organism deficient or lacking in GLA and linoleic acid (LA) which comprises transforming said organism with an isolated nucleic acid encoding bacterial Δ6-desaturase and an isolated nucleic acid encoding Δ12-desaturase.

16. A method of inducing production of gamma linolenic acid (GLA) in an organism deficient or lacking in GLA and linoleic acid (LA) which comprises transforming said organism with at least one expression vector comprising an isolated nucleic acid encoding bacterial Δ6-desaturase and an isolated nucleic acid encoding Δ12-desaturase.

17. The method of any one of Claims 15 or 16 wherein said isolated nucleic acid encoding Δ6-desaturase comprises nucleotides 317 to 1507 of SEQ. ID NO:1.

18. A method of inducing production of octadecatetraeonic acid in an organism deficient or lacking in gamma linolenic acid with comprises transforming said organism with isolated nucleic acid of any one of Claims 1-7.

19. The method of Claim 18 wherein said organism is a bacterium, a fungus, a plant or an animal.

20. A method of use of the isolated nucleic acid of any one of Claims 1-7 to produce a plant with improved chilling resistance which comprises:

a) transforming a plant cell with the isolated nucleic acid of any one of Claims 1-7; and

b) regenerating said plant with improved chilling resistance from said transformed plant cell.

21. The method of Claim 20 wherein said plant is a sunflower, soybean, maize, tobacco, peanut or oil seed rape plant.

22. Isolated bacterial Δ6-desaturase.

23. The isolated bacterial Δ6-desaturase of Claim 22 which has an amino acid sequence of SEQ ID NO:2.

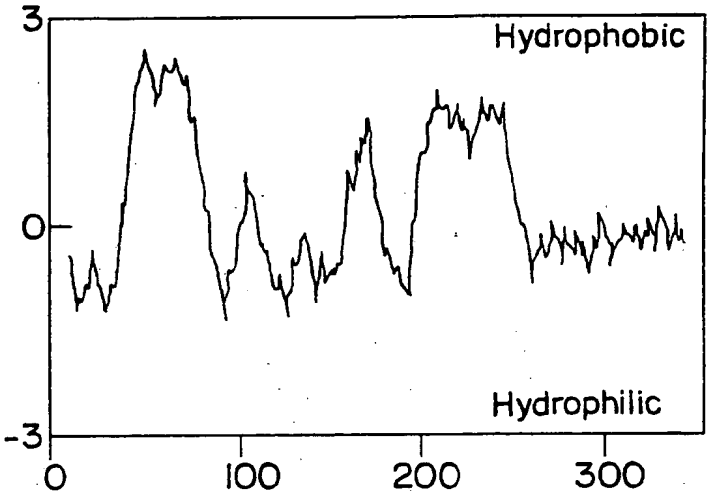

3
0
-3
Hydrophobic
Hydrophilic
0
100
200
300

FIG. IA

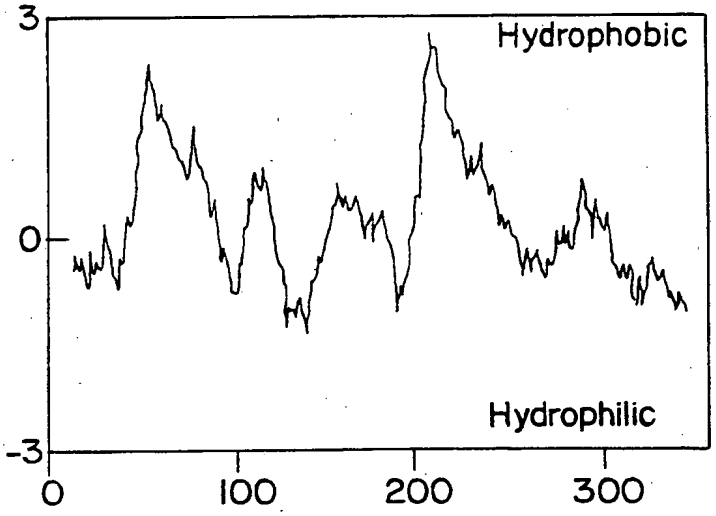

3
0
-3
Hydrophobic
Hydrophilic
0
100
200
300

FIG. IB

FIG. 2A

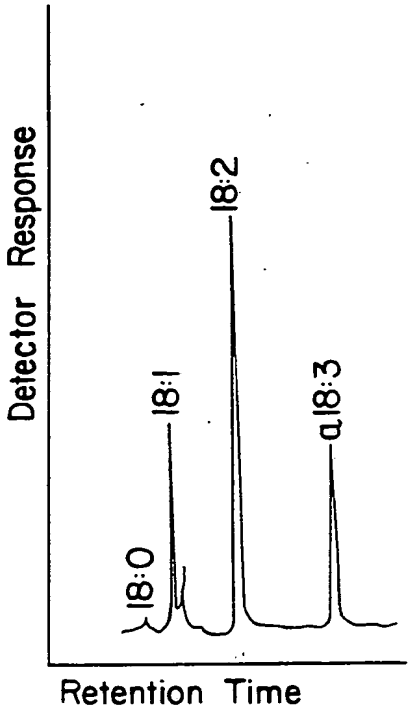
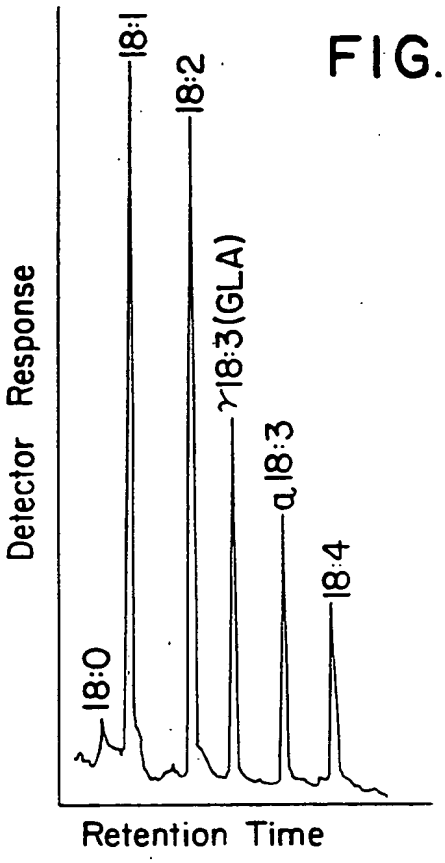


FIG. 2B



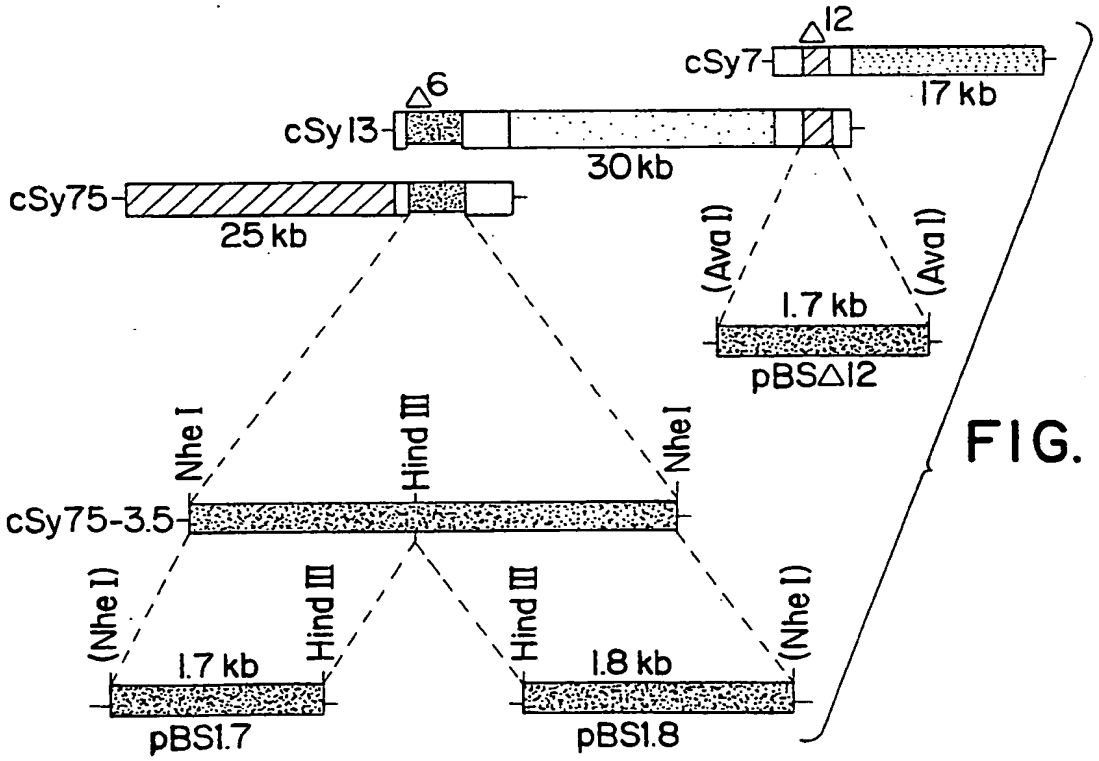


FIG. 3

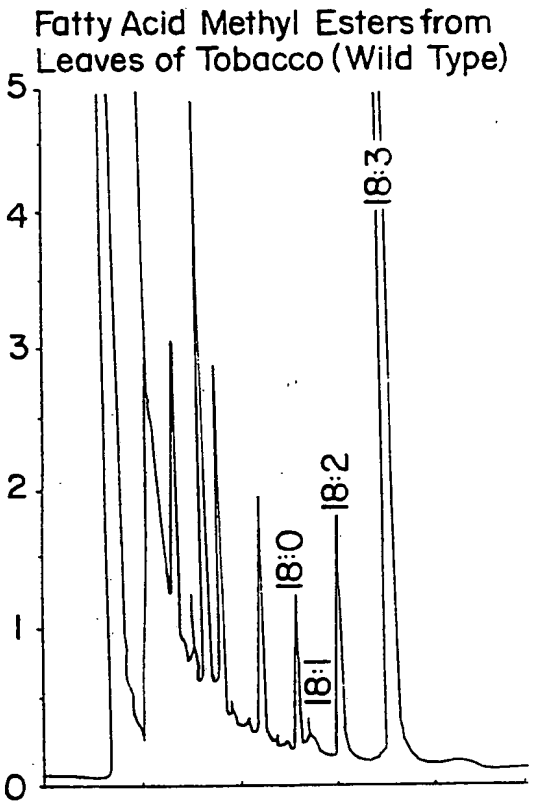

Fatty Acid Methyl Esters from
Leaves of Tobacco (Wild Type)
5
4
3
2
1
0
18:0
18:1
18:2
18:3

FIG. 4A

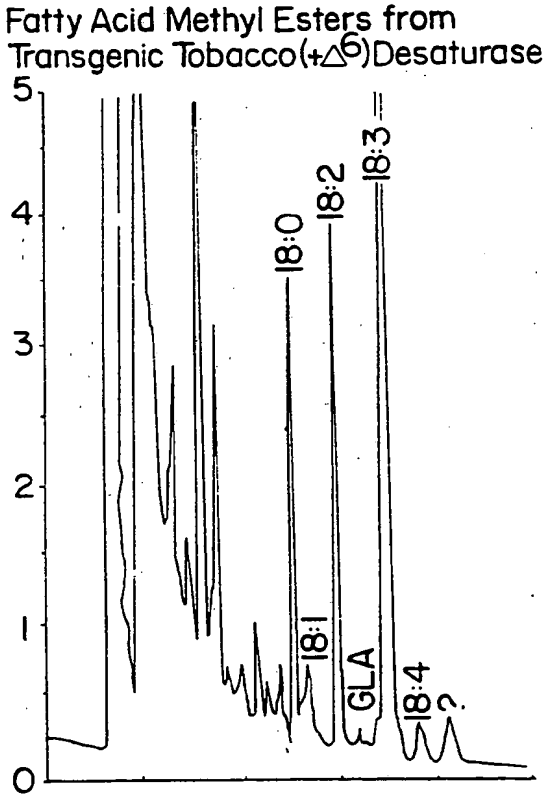

Fatty Acid Methyl Esters from
Transgenic Tobacco(+Δ6)Desaturase
5
4
3
2
1
0
18:0
18:1
18:2
18:3
GLA
18:4
?

FIG. 4B

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US92/08746

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :800/205; 435/172.3, 189, 69.1, 320.1, 134, 170, 171; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205; 435/172.3, 189, 69.1, 320.1, 134, 170, 171; 536/27; 935/9, 30, 6, 24, 29, 38

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN/BIOSIS, CA; APS
search terms: linolenic, desaturase, delta-6, gene, DNA, cDNA, purif?, cyanobacteri?,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 347, issued 13 September 1990, H. Wada et al., "Enhancement of Chilling Tolerance of a Cyanobacterium by Genetic Manipulation of Fatty Acid Desaturation", pages 200-203, especially pages 201-203.	1-23
Y	Biochemical Journal, Volume 240, issued 1986, S. Stymne et al., "Biosynthesis of y-Linolenic Acid in Cotyledons and Microsomal Preparations of the Developing Seeds of Common Borage (Borago officinalis)", pages 385-392, especially pages 385 and 392.	1-23
Y	EP, A, 0,255, 378 (Kridl et al.) 3 February 1988, see entire document, especially columns 3-5 and 7-11.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 DECEMBER 1992	13 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CHARLES C. P. RORIES, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US92/08746

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A01H 1/00, 5/00; C12N 15/00, 9/02; C12P 7/64, 1/02, 1/04, 21/06; C07H 15/12, 17/00

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☒ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLORED OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning documents *will not* correct images problems checked, please do not report the problems to the IFW Image Problem Mailbox